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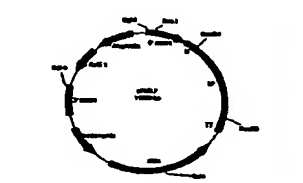
[Continued on next page]

(54) Title: RECOMBINANT YEAST STRAIN FOR EXPRESSING HUMAN LACTOFERRIN, N-LOBE LACTOFERRIN, C-LOBE LACTOFERRIN AND PROCESS FOR PREPARATION RECOMBINANT HUMAN LACTOFERRIN, N-LOBE LACTOFERRIN, C-LOBE LACTOFERRIN USING THE SAME



M: 1kb ladder
Lane 1: pPICLF expression vector
Lane 2: PT (pPICLF expression vector transformant)
Lane 3: pPICLFN expression vector
Lane 4: PN (pPICLFN expression vector transformant)
Lane 5: PC (pPICLFC expression vector transformant)
Lane 6: pPICLFC expression vector
Lane 7: P. pastoris GS115
Lane 8: pPIC9K

(57) Abstract: The present invention relates to a recombinant yeast strain for expressing human lactoferrin, N-lobe lactoferrin, C-lobe lactoferrin and process for preparation recombinant human lactoferrin, N-lobe lactoferrin, C-lobe lactoferrin using the same. The process comprises preparing recombinant vector pPICLF, pPICLFN and pPICLFC respectively for expressing human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin genes by amplifying human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin genes using the PCR method and inserting them to vector pPIC9K; obtaining recombinant yeast strain Pichia pastoris LF2(KCCM-10211), Pichia pastoris NFL3(KCCM-10210) and Pichia pastoris CLF3(KCCM-10212) by transforming auxotroph Pichia pastoris GS115 deficient in histidine dehydrogenase activity using said recombinant vectors; and mass-producing recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin by culturing the said recombinant yeast strains.

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RECOMBINANT YEAST STRAIN FOR EXPRESSING HUMAN LACTOFERRIN, N-LOBE LACTOFERRIN, C-LOBE LACTOFERRIN AND PROCESS FOR PREPARATION RECOMBINANT HUMAN LACTOFERRIN, N-LOBE LACTOFERRIN, C-LOBE LACTOFERRIN USING THE SAME

TECHNICAL FIELD

The present invention relates to recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, recombinant yeast strains able to express the proteins, and the production thereof. More particularly, the present invention is concerned with recombinant yeast strains transformed from Pichia pastoris, which is able to produce a human lactoferrin at a high yield and is easy to regulate in producing the human lactoferrins.

BACKGROUND ART

In association with the history of wine and bread, yeast has made important contributions to human life. As the recent great advances in the life sciences can illuminate the mysteries of life phenomena, the mystery of biometabolism of yeast is also unveiled, as with other organisms. As a result, the biomass of yeast is found to be a good nutritive source for higher organisms as well as being a treasure house in which biochemical materials are abundant. Additionally, the knowledge of the metabolism of yeast allows a variety of useful materials to be produced by taking advantage of it. In fact, yeast is now recognized as being very profitable to the biochemical industry.

Conventionally, E. coli has been extensively used as host cells for the mass production of various physiologically active substances, such as hormones, cell growth factors, cytokines, enzymes, etc., through gene recombinant techniques. However, E. coli is problematic in that it contains endotoxins. Further, the use of E. coli in producing proteins suffers from the drawbacks of

adding a superfluous methionine residue to the N-end of produced recombinant proteins and forming inclusion bodies. In addition, obtained are the recombinant proteins which are produced without undergoing glycosylation, amidation, acylation, phosphorylation, and disulfide binding, so that they are different in tertiary structure from native ones. Difference is also found in folding structure therebetween.

In an effort to avoid the problems, animal cells have been used as host cells. However, in addition to being expensive, animal cells are difficult to culture owing to their relatively long generation cycles. Animal cells require culture media of complex composition and precise equipment for their cultivation, so that the mass production of proteins through them is economically unfavorable.

As an alternative for solving the problems animal cells and E. coli retain, yeast cells have been under intensive study. When producing recombinant proteins, yeast cells are advantageous in the following aspects. First, because yeast is eukaryotic, its cytoplasm provides an environment in which for the recombinant proteins to fold correctly. Next, glycosylation can be performed within yeast, playing an important role in the structural integrity, solubility, and biological activity of proteins. Additionally, yeast is such a non-pathogenic and GRAS (generally recognized as safe) microorganism as can be used in producing foods and medicines. Finally, the extracellular secretion of proteins is possible in yeast, thereby bringing about a great improvement in the purification yield compared with E. coli.

Representative of the yeast strains used for the production of proteins is Saccharomyces. In the case of S. cerevisiae, promoters are preferably present in multi-copy plasmids in order to achieve high expression rates. When this yeast strain is fermented on a large scale, however, the expression rate is readily decreased owing to the loss of the plasmids. Additionally, because promoters derived from glycolytic genes are constitutive, it is difficult to express the genes at

desirable time points. Even controllable promoters are known to have various problems in producing proteins.

To circumvent the problems of Saccharomyces, a second generation yeast expression system was developed using Pichia pastoris, a methylotropic yeast strain. Pichia pastoris not only takes advantage of an AOX_1 promoter, which can precisely control gene expression by methanol inducement with a high expression efficiency, but also is cultured at high concentrations. Under the control of the AOX_1 promoter, an exogenous gene remains in an expression-off state in the presence of only non-methanolic carbon sources. Addition of a methanolic carbon source to the Pichia pastoris culture triggers gene expression. The expression system of P. pastoris has successfully found applications in producing a variety of exogenous proteins. For instance, the expression system of P. pastoris can produce TNF (tumor necrosis factor) at an expression efficiency of 8g/L, tetanus toxin fragment at an expression efficiency of 12 g/L, human serum albumin at an expression efficiency of 4 g/L.

Human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, all 78 kD glycoproteins with three-dimensional bilobal structures (N-lobe and C-lobe) belonging to a transferrin family, are a kind of iron-associated, physiologically active substances and are, for the most part, secreted in the milk of mammalian animals, including humans and found, to a far lesser extent, in the secretions of external fluids, such as tear glands, salivary glands and the prostate gland.

As physiologically active materials, lactoferrins are involved in the control of myelopoiesis in immune systems, the control of inflammatory responses, the production of growth factors necessary for lymphocytes, DNA-binding and the activation of RNases. Additionally, lactoferrins show inhibitory activity against a broad spectrum of enteric putrefaction lawn and play an important role in controlling the absorption of iron to the intestine, serving as a dietary iron source and an antioxidant.

The highest known concentrations of lactoferrin are found in human colostrums, or "first milk", where levels measure as much as 3.4-4.2 mg/mL. A similar pattern exists in bovine (cow) milk, but the levels amounts to as low as 0.02-0.2 mg/mL. Further, the lactoferrin of bovine milk is, for the most part, destroyed during the thermal processing thereof. Therefore, infants who are reared not on breast milk, but with bovine milk, cannot sufficiently enjoy the beneficial effects lactoferrin gives because of deficiency in lactoferrin. Milch cows are liable to frequently suffer from mastitis since the low lactoferrin concentration does not allow the immune system to effectively defend them against the mastitis-causing bacteria. Thus, intensive care should be taken for cows not to contract mastitis, which is correlated with the production of high quality of dairy products.

The first attempt to produce lactoferrin in a large quantity from microorganisms was made by Ward et al., (1992), who applied a gene recombinant technique to Aspergillus oryzae, a kind of fungus. Subsequently, the expression of lactoferrin was studied in Saccharomyces cerevisiae by Liang and Richardson (1993). However, their reports both describe very low expression levels which are in the range from 0.002 to 0.025 mg/mL. Particularly, as for Saccharomyces cerevisiae, this host cell itself is found to be highly susceptible for the lactoferrin produced.

DISCLOSURE OF THE INVENTION

Leading to the present invention, the intensive and thorough research on the production of human lactoferrins, conducted by the present inventors, resulted in the finding that Pichia pastoris is highly suitable as a host cell for recombinant expression vectors as well as being able to produce exogenous proteins at high yields. For this, it is necessary to select an expression vector suitable for the host cell.

Therefore, it is an object of the present invention to provide a recombinant expression vector anchoring a human lactoferrin gene, an N-lobe lactoferrin gene or a C-lobe lactoferrin gene.

It is another object of the present invention to provide a recombinant yeast strain which is able to produce a human lactoferrin at a high yield.

It is a further object of the present invention to provide a recombinant yeast strain which is easy to regulate in producing the human lactoferrins.

It is still a further object of the present invention to provide a method for producing a human lactoferrin, an N-lobe lactoferrin or a C-lobe lactoferrin in a large quantity.

In one aspect of the present invention, there are provided recombinant vectors pPICLF, pPICLFN, and pPICLFC, which are constructed by inserting a human lactoferrin gene, an N-lobe lactoferrin gene and a C-lobe lactoferrin gene into a vector pPIC9K, respectively.

In another aspect of the present invention, there are provided recombinant yeast strains Pichia pastoris LF2 (KCCM-10211), Pichia pastoris NFL3 (KCCM-10210), and Pichia pastoris CLFC3 (KCCM-10212), which are transformed from Pichia pastoris GS115 through the introduction of recombinant vectors pPICLF, pPICLFN, and pPICLFC, which are constructed by inserting a human lactoferrin gene, an N-lobe lactoferrin gene, and a C-lobe lactoferrin gene into an expression vector pPIC9K, respectively.

In a further aspect of the present invention, there are provided recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, which are applicable to feedstuff and medicines.

In a still further aspect of the present invention, there is provided a method for producing the recombinant human lactoferrins, comprising the steps of: culturing the recombinant yeast strains for 24 hours in a 4% glycerol feed batch; subjecting the recombinant yeast strains to preinduction with glycerol for 4

hours; and fermenting a culture medium with the recombinant yeast strains at 30 °C in the presence of methanol.

In still another object of the present invention, there is provided feedstuff, comprising at least one of the recombinant strains and/or at least one of the recombinant lactoferrins.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows schematic diagrams of recombinant expression vectors pPICLF, pPICLFN and pPICLFC, which are prepared by inserting genes for human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin into pPIC9K.

Fig. 2 is a photograph showing agarose gel on which pPICLF (lane 1), a pPICLF transformant (lane 2), pPICLFN (lane 3), a pPICLFN transformant (lane 4), a pPICLFC transformant (lane 5), pPICLFC (lane 6), a P. pastoris GS115 gene (lane 7), and pPIC9K (lane 8) are electrophoresed, along with a 1 kb ladder marker (M).

BEST MODES FOR CARRYING OUT THE INVENTION

The present invention pertains to the mass production of human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin from yeast at high efficiency.

First, it is necessary to construct recombinant vectors capable of expressing the lactoferrin proteins in yeast. Sufficient amounts of cDNA of human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin are obtained by PCR amplification using suitable sets of primers. The amplified PCR products are modified by removing signal sequences therefrom. After being digested with EcoRI, the modified cDNAs for human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin are ligated into pPIC9K which has been linearized with the same restriction enzyme.

Next, there are required host cells capable of harboring the recombinant vectors and expressing the proteins of interest. In this regard, the recombinant vector constructs, designated pPICLF, pPICLFN and pPICLFC, are introduced into E. coli Top10F' and the resulting transformants are cultured to amplify the desired clones. The recombinant expression vectors pPICLF, pPICLFN and pPICLFC are transfected into Pichia pastoris GS115 deficient in histidine dehydrogenase activity. As a result, there are obtained recombinant yeast strains, Pichia pastoris LF2, Pichia pastoris NFL3 and Pichia pastoris CLF3.

In order to determine whether the recombinant yeast strains can express human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, A two-stage selection procedure is performed. First the colonies of the recombinant yeast strains grown on YPD media are cultured in histidine-free media to select His^+ transformants. By taking advantage of the resistance to G418 (geneticin) due to the kanamycin resistance gene of the vector, the screening of the multiple-insert transformants is made.

The next step is to express recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin from transformants. In this connection, SDS-PAGE is useful to determine the expression of recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin. The chromosomal integration of their genes can be identified by PCR. The expression quantities of the recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin can be accurately measured by ELISA. The recombinant yeast strains Pichia pastoris LF2, Pichia pastoris, NFL3, and Pichia pastoris CLF3, which are transformed with the human lactoferrin gene, N-lobe lactoferrin gene and C-lobe lactoferrin gene, respectively, are cultured and, from this primary culture, a supernatant is obtained by centrifugation. The supernatant is used as a material for SDS-PAGE because the proteins are secretive. As a result of 10% SDS-PAGE, the transformed recombinant cells are found to express the recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin. A direct PCR screening technique is useful to

identify the chromosomal integration of genes of interest, that is, the recombinant human lactoferrin gene, N-lobe lactoferrin gene and C-lobe lactoferrin gene. The recombinant yeast strains Pichia pastoris LF2, Pichia pastoris, NFL3, and Pichia pastoris CLF3 are also found to express the targeted proteins as measured by ELISA.

For the mass production of human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, the transformed, recombinant yeast strains, Pichia pastoris LF2, Pichia pastoris NFL3, and Pichia pastoris CLF3 are cultured for 24 hours in a 4 % glycerol feed batch. The production is easily regulated through preinduction with glycerol and induction with methanol.

Along with the cell mass, the recombinant human lactoferrin, N-lobe lactoferrin, and C-lobe lactoferrin separated from the culture by centrifugation are useful as additives to prepare feedstuff.

The genes for human lactoferrin, N-lobe lactoferrin, and C-lobe lactoferrin, used in the present invention, are 2.1 kb in size, obtained from Lab. of Dairy Science & Lactation Physiology, Dept. of Animal Science & Technology, at College of Agriculture & Life Sciences, Seoul National University, Korea.

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

EXAMPLE 1

Construction of Recombinant Expression Vectors for Human Lactoferrin, N-lobe Lactoferrin and C-lobe Lactoferrin and Transformation of Yeast Strain therewith

First Step: Selection of host cells for the production of human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin

After scrutiny of references from KCTC and ATCC and other documents, Pichia pastoris GS115, which is deficient in histidine dehydrogenase activity, was selected as a test strain. This histidine auxotroph was cultured in a complex medium such as YPD or a MM (minimal methanol) or MD (minimal dextrose) medium supplemented with histidine at 30 °C with shaking or in a plating manner.

Second Step: PCR amplification of genes of human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin

Human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin gene cDNAs, all from the lab of the present inventors, were amplified by PCR with the strategy of making respective genes free of signal sequences. In regard to this PCR amplification, primers were synthesized to have the following sequences:

Primers for PCR amplification of human lactoferrin

EcoRI

HLF-N: 5'-CAACGGAATTCGGCCGTAGGAGAAGGAGTG-3' (30mer)

HLF-C: 5'-CAACGGAATTCTTTACTTCCTGAGAAACTCACAGGC-3'(36mer)

Primers for PCR amplification of N-lobe lactoferrin

EcoRI

NLF-N: 5'-CAACGGAATTCGGCCGTAGGAGAAGGAGTG-3' (30mer)

NLF-C1:5'-CAACGGAATTCTTTACACCCTCGAAAACCCAATGGC-3'(36mer)

Primers for PCR amplification of C-lobe lactoferrin

EcoRI

NLF-N1: 5'-CAACGGAATTCGCCCGGCGTGCGCGGG-3' (27mer)

HLF-C : 5'-CAACGGAATTCTTTACTTCCTGAGAAACTCACAGGC-3'(36mer)

As seen in the base sequences, each primer was designed to contain a EcoRI recognition site via which the PCR product could be inserted into a pPIC9K vector. In addition, as for primers for human lactoferrin and C-lobe lactoferrin genes, they were modified in order to produce PCR products lacking the EcoRI recognition site that human lactoferrin and C-lobe lactoferrin genes originally contain. In this regard, the base sequence of the EcoRI recognition site was changed to GAAACTC as underlined in the primer HLF-C. PCR conditions included denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 130 sec with last extension for additional 10 min. The PCR product was identified by agarose gel electrophoresis.

Third Step: Construction of expression vector

After being treated with EcoRI, the PCR-amplified genes for human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin were separately ligated to pPIC9K vector which was previously cut with the same restriction enzyme. The ligation products thus obtained were separately transfected into TOP10F. From the transformants were obtained recombinant vectors capable of expressing human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, which were designated "pPICLF", "pPICLFN", and "pPICLFC", respectively. Their structures are schematically shown in Fig. 1.

Fourth Step: Transformation of Pichia pastoris

The aim of this step was to create a His^+Mut^+ Pichia strain. To this end, the recombinant vectors pPICLF, pPICLFN and pPICLFC were cut with SalI. Thereafter, the linearized DNAs were integrated into the HIS region of Pichia to

give the desired strain. To begin with, Pichia pastoris GS115 (His⁻ mutant) was cultured overnight in 50 mL of a YPD medium at 30 °C, after which 0.1-0.5 mL of the culture was inoculated in 500 mL of YPD in a 2 L flask and incubated to an OD_{600} of 1.3-1.5. After centrifugation at 1,500xg at 4 °C for 5 min, the pellet was dissolved in 500 mL of cold sterilized deionized water. Re-centrifugation under the same conditions was followed by the dissolution of the pellet in 250 mL of cold sterilized deionized water. The same procedure was repeated with 20 mL and 1 mL of 1 M sorbitol to give competent cells at a final volume of 1 mL. 80 μL of the competent cell was mixed with 5-20 μg of each of the recombinant vectors pPICLF, pPICLFN and pPICLFC linearized with SalI and incubated in an electroporation cuvette for 5 min in ice, followed by applying an electric field of 1,500 volt, 200 Ω across the cuvettes to transfect the linearized DNAs into the competent cells. Immediately after the electroporation, the competent cells in the cuvettes were added with 1 mL of 1 M sorbitol and transferred to sterilized microcentrifuge tubes. On MD plates were spread the cells which were then cultured at 30 °C until colonies appeared.

Fifth Step: Selection of Pichia pastoris transformants

A two-stage selection procedure was performed to determined whether the colonies formed in the fourth step were transformed with the vectors. Because the Pichia strain used for the transformation is a His⁻ mutant, the colonies, if transformed, had been His^+ strains; if not transformed, they were still His deficient. Therefore, the competent cells were grown on histidine-free MD (minimal dextrose medium-histidine; 1.34% YNB, $4x10^{-5}$% biotin, 2% dextrose) plates to select His^+ strains. In the next selection stage, advantage was taken of the resistance to G418 (geneticin) due to the kanamycin resistance gene of the vector in screening multiple-insert transformants. YPD-G418 plates containing various concentrations (0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0 and 4.0 mg/ml) of G418 were prepared. 1-2 mL of sterilized deionized water was dropped onto

His$^+$ transformant colonies grown on each plate, followed by resuspending the transformant colonies with the aid of a sterile spreader. Suspensions of the cells were transferred into 50 mL centrifuge tubes, vortexed and measured for cell density by use of a spectrophotometer. $1x10^5$ cells were plated over the YPD-G418 plates with various concentrations of G418. Incubation at 30 °C made colonies appear on the plates. Colonies formed on YPD-G418 plates with higher concentrations of G418 were found to have greater copy numbers.

Sixth Step: Expression of recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin from transformants

In this step, determination and identification was made for the expression of recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin by SDS-PAGE, for the chromosomal integration of their genes by PCR and for the expression quantities thereof by ELISA.

To begin with, recombinant yeast strains Pichia pastoris LF2, Pichia pastoris, NFL3, and Pichia pastoris CLF3, which were transformed with the human lactoferrin gene, N-lobe lactoferrin gene and C-lobe lactoferrin gene, respectively, were cultured for 72 hours in MM induction media and 1 mL of each of the cultures was centrifuged at 5,000 rpm for 5 min. The supernatant was concentrated to 10 μL whose proteins were separated by 10% SDS-PAGE. The proteins were visualized as bands by dying with coommassie brilliant blue to determine whether the recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin were expressed and secreted.

Next, success in the chromosomal integration of genes of interest was determined by PCR. In this connection, a direct PCR screening technique was employed. In a 1.5 mL centrifuge tube, either 10 μL of a Pichia pastoris culture or a mixture of 1 μL of the culture and 9 μL of sterile deionized water was added, or a single colony was suspended in 10 μL of sterile deionized water. The addition of 5 μL of lyticase (5 U/μL) was followed by the incubation at 30 °C for

10 min. The samples were chilled at –70 °C for 10 min and used as templates for PCR with 5' AOX_1 primer and 3' AOX_1 primer. 5 μl of Taq polymerase (0.15 U/μL) was added to a PCR pre-mix after the denaturation thereof at 95 °C for 5 min, followed by conducting thermal cycles, each consisting of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min and for additional 5 min for the last extension. DNAs amplified by the PCR were found to be 2.2 kb and 2.5 kb in size as measured by agarose gel electrophoresis. These sizes of the gene bands were coincident with those of wild-type AOX_1 gene (2.2 kb) and human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin (all 2.5 kb).

To measure the quantities of the expressed human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, ELISA was conducted. ELISA is so highly sensitive as to cover a detection range from 0.2 to 200 μg/L and so fast as to finish the measurement within about 7 hours in addition to being able to treat a number of samples, simultaneously. Antibodies against human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin were coated onto 96-well plates. For this, the antibodies were dissolved at a concentration of 1.0 μg/mL in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) and each solution was added at a volume of 150 μL per well and incubated at 37 °C for 2 hours, followed by washing the plates. A

lactoferrin standard (0.2-200 ng/mL) or samples were added to the antibody-coated 96-well plates in tripartition at an amount of 100 μL (1 μg/mL) per well. Incubation at 37 °C for 1 hour was followed by washing. After the addition of 100 μL of an avidin-peroxidase conjugate (1/100,000 dilution) per each well, incubation at 37 °C for 1 hour was conducted in advance of washing. 100 μL of an enzyme substrate was reacted at 37 °C for 20 min with the conjugated enzyme, after which absorbance at 490 nm was measured to determine quantities of the recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin.

Recombinant yeast strains Pichia pastoris LF2, Pichia pastoris NFL3 and Pichia pastoris CLF3, which harbored expression vector constructs at which genes for human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin were anchored, respectively, were deposited in Korean Culture Center of Microorganisms, on Oct. 18, 1999, under the deposition Nos. KFCC-11106, KFCC-11107 and KFCC-11105, which were, respectively, converted into International Deposition Numbers KCCM-10211, KCCM-10210 and KCCM-10212 on Sep. 2, 2000, in accordance with the Budapest Treaty.

EXAMPLE 2

Fermentation of Pichia pastoris for Mass Production of Recombinant Human Lactoferrin, N-Lobe Lactoferrin and C-lobe Lactoferrin

Media necessary for the mass production of the recombinant lactoferrins were used as shown in Table 1, below. Transformed, recombinant yeast strains, Pichia pastoris LF2, Pichia pastoris NFL3, and Pichia pastoris CLF3 were separately inoculated in a medium in a 5 L fermenter and cultured for 24 hours in a 4 % glycerol feed batch. After preinduction for 4 hours with glycerol, the transformed, recombinant yeast strains were subjected to induction with methanol for 72 hours in total, to produce recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin. The fermentation was carried out at an air flow rate of 1

vvm (volume of air per volume of medium per minute) at 30 °C under a stirring condition of 1,000 rpm while the pH was controlled within the range of 5.0±0.05 with ammonia water. One of the colonies of Pichia pastoris LF2, Pichia pastoris NFL3 or Pichia pastoris CLF3 was inoculated in 20 mL of a flask medium in a 100 mL baffle flask and cultured for 24 hours at 30 °C with shaking. In a first scale-up stage, 5 mL of the resulting culture (primary seed culture) was aseptically inoculated in 100 mL of a flask medium in a 500 mL baffle flask and cultured at 30 °C for 16 hours with shaking. Next, 100 mL of the resulting culture (secondary seed culture) was transferred to a 5 L fermenter containing 2 L of a basal medium which was previously autoclaved and controlled to pH 5.0 with ammonia water, followed by performing fermentation with an additional supply of a batch-fed carbon source. The fermentation conditions included an air flow rate of 1 vvm, a stirring rate of 1,000 rpm, a temperature of 30 °C, and a pH of 5.0.

TABLE 1

Media for Fermentation of Recombinant Yeast Strains Pichia pastoris LF2, Pichia pastoris NFL3 , Pichia pastoris CLF3

Flask medium	1.34% YNB, 1% glycerol, $4.\times10^{-5}$ biotin
Basal medium	Phosphoric acid 85% 26.7mL, calcium sulfate 0.93g, potassium sulfate 18.2g, magnesium sulfate-$7H_2O$ 14.9g, potassium hydroxide 4.13g, C-source 40.0g, PTM solution 4.35ml per liter.
PTM solution	cupric sulfate-$5H_2O$ 6.0g, potassium iodide 0.08g, manganese sulfate-H_2O 3.0g, sodium molybdate-$2H_2O$ 0.2g, boric acid 0.02g, cobalt chloride 0.5g, zinc chloride 20.0g, ferrous sulfate-$7H_2O$ 65.0g, biotin 0.2g, sulfuric acid 5.0mL per liter.
Feeding medium	50% glycerol, 6mL PTM solution
Induction medium	100% methanol, 12mL PTM solution

EXAMPLE 3

Preparation of Feedstuff Containing Recombinant Human Lactoferrin, N-Lobe Lactoferrin and C-Lobe Lactoferrin

The recombinant human lactoferrin, N-lobe lactoferrin, and C-lobe lactoferrin obtained through the fermentation in Example 2 were used, along with the cell mass, as additives to prepare feedstuff. In this regard, after the fermentation, the culture was separated into a supernatant and cell mass by centrifugation. The supernatant was freeze-dried to obtain recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin, which was added, along with the cell mass also freeze-dried, at an amount of 0.1-0.5 % by weight based on the total weight of feedstuff comprising, for example, soybean oil cake, bean, etc.

INDUSTRIAL APPLICABILITY

As described hereinbefore, recombinant yeast strains Pichia pastoris LF2, Pichia pastoris NFL3 and Pichia pastoris CLF3 are provided for producing recombinant human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin in a large quantity. The recombinant yeast strains capable of expressing the proteins of interest can be prepared by transforming auxotrophic Pichia pastoris GS115 deficient in histidine dehydrogenase activity with recombinant expression vectors pPICLF, pPICLFN and pPICLFC, which are constructed by inserting a human lactoferrin gene, a human N-lobe lactoferrin gene and a human C-lobe lactoferrin gene into pPIC9K, respectively. Culturing the recombinant expression yeast strains in suitable media yields the proteins of interest, that is, human lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin. When they are added, along with the recombinant yeast strains, to feedstuff, it is very useful to improve the efficiency of feedstuff. With natural antibiotics activity against harmful enteric bacteria,

the recombinant lactoferrin, N-lobe lactoferrin and C-lobe lactoferrin can find various applications in the biomedical industry and the livestock industry.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

CLAIMS

1. A recombinant vector pPICLF, consisting of pPIC9K as a base vector and a human lactoferrin gene as an insert.

2. A recombinant yeast strain Pichia pastoris LF2 (KCCM-10211), transformed from Pichia pastoris GS115 through the introduction of a recombinant vector pPICLF, said pPICLF being constructed by inserting a human lactoferrin gene into an expression vector pPIC9K.

3. A recombinant human lactoferrin, obtained by culturing the recombinant yeast strain of claim 2, Pichia pastoris LF2 (KCCM-10211).

4. A method for producing the recombinant human lactoferrin of claim 3, comprising the steps of:

culturing the recombinant yeast strain of claim 2, Pichia pastoris LF2 (KCCM-10211) for 24 hours in a 4% glycerol feed batch;

subjecting the recombinant yeast strain to preinduction with glycerol for 4 hours; and

fermenting a culture medium with the recombinant yeast strain at 30 °C in the presence of methanol.

5. A recombinant vector pPICLFN, consisting of pPIC9K as a base vector and an N-lobe lactoferrin gene as an insert.

6. A recombinant yeast strain Pichia pastoris NFL3 (KCCM-10210), transformed from Pichia pastoris GS115 through the introduction of a recombinant vector pPICLFN, said pPICLFN being constructed by inserting an N-lobe lactoferrin gene into an expression vector pPIC9K.

7. A recombinant N-lobe lactoferrin, obtained by culturing the recombinant yeast strain of claim 6, Pichia pastoris NFL3 (KCCM-10210).

8. A method for producing the recombinant human lactoferrin of claim 6, comprising the steps of:

culturing the recombinant yeast strain of claim 6, Pichia pastoris NFL3 (KCCM-10210), for 24 hours in a 4% glycerol feed batch;

subjecting the recombinant yeast strain to preinduction with glycerol for 4 hours; and

fermenting a culture medium with the recombinant yeast strain at 30 °C in the presence of methanol.

9. A recombinant vector pPICLFC, consisting of pPIC9K as a base vector and a C-lobe lactoferrin gene as an insert.

10. A recombinant yeast strain Pichia pastoris CLFC3 (KCCM-10212), transformed from Pichia pastoris GS115 through the introduction of a recombinant vector pPICLFC, said pPICLFC being constructed by inserting a C-lobe lactoferrin gene into an expression vector pPIC9K.

11. A recombinant N-lobe lactoferrin, obtained by culturing the recombinant yeast strain of claim 10, Pichia pastoris CLF3 (KCCM-10212).

12. A method for producing the recombinant human lactoferrin of claim 10, comprising the steps of:

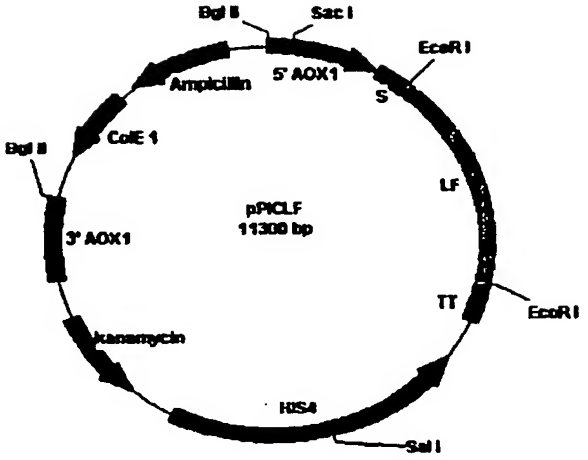
culturing the recombinant yeast strain of claim 10, Pichia pastoris CLF3 (KCCM-10212), for 24 hours in a 4% glycerol feed batch;

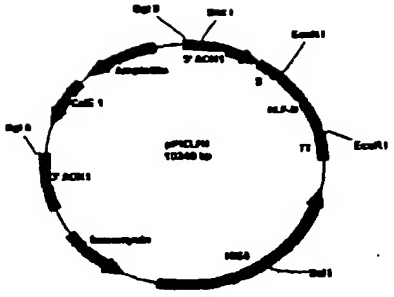
subjecting the recombinant yeast strain to preinduction with glycerol for 4 hours; and

fermenting a culture medium with the recombinant yeast strain at 30 °C in the presence of methanol.

13. Feedstuff, comprising the recombinant strains of claims 2, 5 and/or 10 and/or the recombinant lactoferrins of claims 3, 7 and/or 11.

Fig. 1





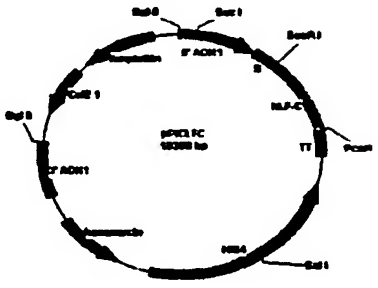
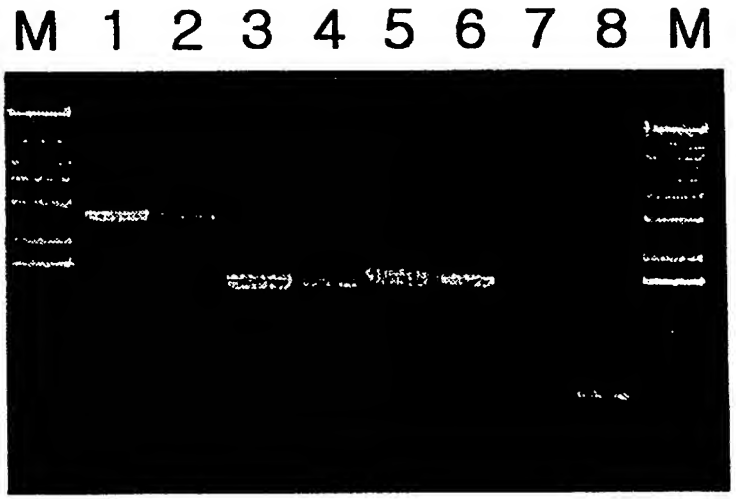


Fig. 2



M: 1kb 1adder

Lane 1: pPICLF expression vector

Lane 2: PT (pPICLF expression vector transformant)

Lane 3: pPICLFN expression vector

Lane 4: PN (pPICLFN expression vector transformant)

Lane 5: PC (pPICLFC expression vector transformant)

Lane 6: pPICLFC expression vector

Lane 7: P. pastoris GS115

Lane 8: pPIC9K

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/01040

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC 7. C12N

Documentation searched other-than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the international search (name of data base and. where practicable, search trerms used)

IBM patent database. NCBI pubmed. "lactoferrin. production. yeast"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
A	US 5571896 (Baylor college of medcine) Nov 5. 1996	1-13
A	US 5571691 (Baylor college of medcine). Nov 5. 1996	1-13
A	US 5766939 (Baylor college of medcine). Jun. 16. 1998	1-13
A	US 58498881 (Baylor college of medcine). Dec. 15. 1998	1-13
P	US 6066469 (Ferro dynamics Inc). May 23. 2000	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure. use. exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 MARCH 2001 (22.03.2001)	22 MARCH 2001 (22.03.2001)

Name and mailing address of the ISA/KR	Authorized officer
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